

THE FUNCTION OF BILE SALTS IN FAT ABSORPTION

by

Alan F. Hofmann

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From the Department of Physiological Chemistry of the University of Lund, Sweden
Chief: Professor Bengt Borgström, M.D.

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This thesis is presented with due permission of the Medical Faculty, and will be publicly discussed in English in the Lecture Hall of the Department of Physiological Chemistry on September 25th at 9:15 A.M., for the degree of Doctor of Medicine.



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This dissertation consists of the following papers, as well as the affixed discussion and summary. The papers are referred to in the text by the appropriate Roman numerals, e.g. Paper II.

- I Thin-Layer Adsorption Chromatography of Lipids.
Biochemical Problems of Lipids (Editor: A.C. Frazer). BBA Library 1.
Elsevier Publishing Company, Amsterdam, 1963, p. 1.
- II The Preparation of Chenodeoxycholic Acid and Its Glycine
 and Taurine Conjugates.
Acta Chemica Scandinavica 17: 173, 1963.
- III The Function of Bile Salts in Fat Absorption: The Solvent
 Properties of Dilute Micellar Solutions of Conjugated
 Bile Salts.
Biochemical Journal 89: 57, 1963.
- IV The Behavior and Solubility of Monoglycerides in Dilute,
 Micellar Bile-Salt Solution.
Biochimica et Biophysica Acta 70: 306, 1963.
- V Hydrolysis of Long-chain Monoglycerides in Micellar Solution
 by Pancreatic Lipase.
Biochimica et Biophysica Acta 70: 317, 1963.
(together with B. Borgström)
- VI The Intraluminal Phase of Fat Digestion in Man: The Lipid
 Content of the Micellar and Oil Phases of Intestinal
 Content Obtained During Fat Digestion and Absorption.
Journal of Clinical Investigation 43: 247, 1964.
(together with B. Borgström)
- VII The Site of Absorption of Conjugated Bile Salts in Man
Gastroenterology 45: 229, 1963.
(together with B. Borgström and G. Lundh)

INTRODUCTION

This dissertation deals with the physico-chemical state of lipids in the small intestinal lumen during fat digestion and absorption. Dietary triglyceride is rapidly and extensively hydrolyzed by pancreatic lipase to fatty acids and partial glycerides (for reviews, see Borgström, 1960; Senior, 1964) and it is the state of these lipids in intestinal content which is the major concern of this work. These lipids would be essentially insoluble in intestinal content were it not for the presence of bile salts (Moore and Rockwood, 1897; Hofmann, 1961). Bile salts are amphipathic substances (for definition, see Hartley, 1936; Bangham, 1963) which completely alter the behaviour of these lipids in aqueous solution. The work described here is perforce concerned with the interaction of bile salts and fatty acids and glycerides, or from a broader point of view, some functions of bile salts in fat digestion and absorption.

Previous advances in four areas of scientific knowledge have made this work possible. The first, largely conceptual, was the elucidation of the physico-chemical properties of detergent solutions. This work began with the discovery of the micelle by McBain (1913), was given great impetus by the brilliant insight of Hartley (1936, 1955), and is continuing actively (Shinoda, 1963; McBain and Hutchinson, 1955; Durham, 1961). The second, methodological, was the development of techniques for the preparation, purification, and quantification of bile salts, notably by the school of Bergström, especially his colleagues Norman (1955) and Sjövall (1964). The third was the understanding of the chemical events in pancreatic lipolysis and fat absorption (Borgström, 1960; Mattson and Beck, 1956; Desnuelle and Savary, 1963). The fourth was the demonstration that the behaviour of model systems of soap, water, and a water-insoluble but polar lipid could be described by conventional phase diagrams and that understanding of such model systems was valuable for the interpretation of the phase equilibria existing in the intestinal lumen. The Department of Chemistry at the University of Scheffield, England, directed by A.S.C. Lawrence, has worked actively in this area for the past decade; its contributions are fundamental (Lawrence and Stenson, 1957; Lawrence, 1959, 1961).

Part I is a brief review of the chemistry of bile acids and their conjugates. It was necessary to prepare large quantities of bile salts and the techniques involved are outlined. Part II discusses the micellar aggregation of bile salts and detergents. Part III describes the behaviour and solubility of the physiologically important solutes, fatty acids, mono-, di-, and triglycerides, in bile salt solutions. These experiments showed that di- and triglycerides possessed a negligible solubility in bile salt solutions, but that under appropriate experimental conditions, fatty acids and monoglycerides had a high micellar solubility. Monoglyceride in micellar solution was used as substrate for pancreatic lipase. These experiments are described briefly.

The results of the solubility studies described in Part III allowed the prediction that if a micellar phase occurred in intestinal content during fat digestion, its lipids should be restricted largely to fatty acid and monoglyceride. Part IV presents analyses of human small intestinal content obtained during fat digestion and absorption, which indicated that a micellar phase containing bile salt, fatty acid, and monoglyceride occurs during fat digestion, as was predicted from the in vitro studies. Part V presents additional experiments which suggest that in man, just as in other mammals, net bile salt absorption does not occur at the same intestinal site as fat absorption, but more distally.

I. Chemistry of Bile Acids and Their Conjugates

A. SIGNIFICANCE OF CONJUGATION.

In man and other mammals, the bile acids in bile are conjugated in peptide linkage with glycine or taurine (see Haslewood, 1955, 1962), and free bile acids are not present. The identification of glycine and taurine conjugates in bile was established over a century ago, but the recognition that the free bile acid moiety obtained by alkaline hydrolysis comprised a number of different compounds did not occur until some years later (for review, see Sobotka, 1935).

Verzar and Kuthy (1929) noted that a solution of fatty acid, sodium deoxycholate, and sodium cholate became turbid when acidified to pH 7, but a solution of the same fatty acid and sodium glycocholate or sodium taurocholate could be acidified to pH 6 before turbidity occurred. The same type of experiments were performed by incubating dilute solutions of the sodium salts of bile acids with buffers of differing pH (Hofmann, 1961). The following was observed: taurine conjugates were soluble at any pH; glycine conjugates crystallized from solution below a pH of about 5; sodium cholate was metastable at pH 6.3 and eventually crystallized from solution; and sodium deoxycholate and sodium chenodeoxycholate precipitated from solution below pH 7 (see also Rich and Blow, 1958). The exact pH at which precipitation occurs is known to be influenced by a number of factors (Ekwall, Lindström and Setälä, 1951; Paper III). The significant conclusion from these experiments was that the glycine and taurine conjugates were soluble at the relatively acid pH (pH 6.0-6.5) generally present in human small intestinal lumen during fat absorption (Borgström, Dahlqvist, Lundh and Sjövall, 1957) but the free bile acids were not.

A concept of the significance of conjugation was suggested which compared the biological process of conjugation to the industrial process of modifying the polar end of a fatty acid or alkanol in order to prepare a detergent with a low pK_a (Hofmann and Borgström, 1962). In the Igepon detergents, for example, a fatty acid is linked by a peptide linkage to N-methyl taurine; such derivatives are comparable to the taurine conjugated bile salts. The glycine or sarcosine conjugates of fatty acids have found less industrial use, although lauryl sarcosinate is a detergent

used as a popular toothpaste additive (Gardol). Van den Oord, Danielsson, and Ryhage (1964) have recently isolated a fatty acid sarcosinate from the crab intestine, suggesting that invertebrates have found a very reasonable solution to the problem of an acid-resistant intestinal detergent. Alkyl sulfates (sulfated alkanols) are the major components of certain household detergents. The analogous derivative occurs in nature in the shark, where the bile alcohol scymol occurs in bile as a sulfate.

Conjugation with glycine or taurine is but one of many ways in which the free bile acids could be converted to an anionic detergent with a low pK_a , but no other types of conjugation have been found. Ornithine conjugates, which are zwitterionic, have recently been reported (Peric-Golia and Jones, 1963; Poley, Dower, Owen, and Strickler, 1964) but their significance is unknown.

B. PURIFICATION OF FREE AND CONJUGATED BILE ACIDS.

1. General considerations:

With present fractionation techniques, it is quite difficult to isolate and purify a conjugated bile acid from bile. Countercurrent distribution can achieve a considerable purification, but the procedure is time-consuming and expensive. Adsorption chromatography gives only a limited resolution of the conjugates, because the polar moiety of the molecules influences the chromatographic mobility much more strongly than the hydroxy-groups. The compounds are not suitable for preparative gas-liquid chromatography. Therefore, when a pure conjugate is needed, it is advisable to prepare the free bile acid in pure form and then to conjugate this with glycine or taurine. Isolation of the pure conjugate from the reaction mixture is not difficult.

2. Thin-layer chromatography of bile acids.

The purity of a bile acid is best assayed chromatographically. Determination of the melting point is of limited value because of the frequent occurrence of polymorphic forms with widely differing melting points (Norman, 1955; Paper II), the tendency of bile acids to form mixed crystals (Wieland and Kishi, 1932) or inclusion compounds (Sobotka, 1935), and the tendency of bile acids to co-crystallize with solvent molecules. Because of the difficulty in eliminating all solvent from crystals, determination

of the equivalent weight is hazardous. Both elemental analysis and optical rotation are insensitive; both give values which represent the algebraic sum of all impurities present.

Paper chromatography has been valuable for the analysis of bile acids and continues to find general application (Sjövall, 1964). Thin-layer chromatography is rapidly replacing paper chromatography for qualitative analysis, not because thin-layer chromatography is especially suitable for bile acid separation, but rather because of its speed of separation, convenience, sensitivity, and capacity (Stahl, 1962).

A method was developed for applying a uniform layer of the adsorbent to microscope slides or small square glass plates (Hofmann, 1962) allowing the rapid screening of numerous solvent combinations. A solvent system was developed which gave very satisfactory resolution of a number of free bile acids, although the chenodeoxycholic-deoxycholic separation was not complete (Paper I), but see Hamilton, (1963). Subsequently the thin-layer chromatographic behaviour of a large number of bile acids and their derivatives has been examined in some detail (Eneroth, 1963; Hofmann, 1964a). A solvent system which separated conjugated bile acids according to class was composed and has found extensive use in this laboratory (Paper I).

A discussion of hydroxyapatite as a thin-layer chromatographic adsorbent for protein, lipid, and bile acid separations was published (Hofmann, 1964a). The isomeric 1- and 2-monoglycerides are resolved on hydroxyapatite; such a separation seems impossible to obtain on silicic acid.

Thin-layer chromatography was used for the evaluation of purity of free bile acids and conjugates prepared synthetically (see above). Chromatographic homogeneity does not necessarily indicate compound purity, for thin layer separations are based largely on the number and arrangement of polar groups. When indicated, two dimensional chromatography or chromatography with several solvent systems was carried out.

3. Preparation of pure unconjugated bile acids.

When the amounts required are small and when the contaminants differ in chromatographic mobility, it is simplest to chromatograph the methyl ester of the desired compound on alumina. Columns of Woelm alumina, inactivated with water to the desired grade, and eluted (stepwise or with

a gradient) with ethyl acetate-benzene or acetone-benzene mixtures have proved quite satisfactory (Neher, 1964). However, as large amounts of the bile acids occurring in man were needed, methods for purifying cholic acid and deoxycholic acid by crystallization were developed. The general procedure was to crystallize a known weight of material in a known volume of solvent and determine semiquantitatively the ratios of impurities present in the crystals and mother liquor by thin layer chromatography. The following crystallization procedures were developed for acids obtained from the commercial sources indicated. It cannot be assumed that these procedures will prove equally satisfactory with other brands.

Cholic acid (Riedel de Haen, Hanover, Germany). 100 gm. cholic acid is refluxed (several hours) in 1200 ml. absolute ethanol until dissolved; 2000 ml. petroleum ether is added. After 24 hours at room temperature (occasional stirring), the suspension is filtered. Yield: 90%. Purity: estimated at greater than 99%.

Deoxycholic acid (T. Schuchardt, München, Germany). 50 gm. is crystallized from 800 ml. aqueous acetone (640 ml. acetone plus 140 ml. water). Yield: 80%. Purity: 97-98%. Both methyl cholate and methyl deoxycholate are readily crystallized from methanol.

Chenodeoxycholic acid is now available commercially, but it is expensive. The conversion of cholic acid to chenodeoxycholic acid was studied in some detail, and the previous synthetic procedures (Fieser and Rajagopalan, 1950; Anderson, Haslewood, Wiggins, and Wootton, 1952) modified so that a pure product could be prepared in a simple and reproducible manner (Paper II). It was found that chenodeoxycholic acid exists in two polymorphic forms: the first, prisms from ethyl acetate-heptane, has a m.p. of 119°; the second, generally a gel obtained from ethyl acetate has a m.p. of 146-148°, after drying. Wieland and Reverey (1924) had observed feathery crystals of chenodeoxycholic acid with a m.p. of 105-110°, but their observations are quoted infrequently and most workers have isolated only the higher m.p., solvent-rich aggregate with m.p. from 142 to 148, depending on its purity. The lower m.p. form is isolated only when the acid is extremely pure; it seems to represent the true crystalline form of chenodeoxycholic acid.

4. The conjugation step; purification of the reaction mixture.

The application of the mixed carboxylic-carbonic anhydride method of peptide synthesis to the preparation of conjugated bile acids (Bergström and Norman, 1953; Norman, 1955) was a great advance. Since pure unconjugated bile acids can be isolated as described above, since side reactions during the reaction are few, and since the procedure usually conjugates about 70-80% of the bile acid, the only problem is the isolation of the pure conjugate from the reaction mixture. The major impurities are glycine or taurine, salt, tri-n-butylamine, and unreacted, free bile acid. The purification of each conjugate differs, and the procedures which have been used to prepare the compounds employed in these experiments are described in the accompanying flow sheets. The conjugation step is carried out as described by Norman (1955) except that dioxane-methylal mixtures or methylal alone (rather than dioxane) were found to be preferable solvents because of their lower m.p. For the numerous evaporation steps, a rotary evaporator with an adjustable air leak is probably essential. The final product obtained by freeze drying is a free-flowing, non-hygroscopic powder. Derivatives of taurocholate and taurochenodeoxycholate which crystallize readily are needed.

Reversed phase partition chromatographic methods for the purification of bile acid conjugates have been described by Norman (1953). The glycine conjugates can also be chromatographed as their methyl esters on columns of inactivated silicic acid (Paper II). This procedure is of value because the glycine conjugates, when prepared by the method of Norman, may be contaminated by more polar impurities which are not removed by the crystallization procedures described in the flow sheet. A batch chromatographic procedure for the purification of glycodeoxycholate and glycochenodeoxycholate is described in the flow sheet.

Flow sheet for synthesis of taurine conjugates of cholic acid, deoxycholic acid, and chenodeoxycholic acid.

- 1) Perform conjugation as described by Norman (1955). Use dioxane-methylal (4:1, by vol.) as solvent. Add molecular sieves prior to addition of ethyl chloroformate. Carry out synthesis in a round bottomed flask.
- 2) Concentrate reaction mixture to thick syrup on rotary evaporator.
- 3) Dissolve syrup in 60% aqueous ethanol. Neutralize (NaOH) to obtain solution.
- 4) Filter solution through sintered glass funnel (Buchner type) to remove molecular sieves and unreacted taurine.

- 5) Adjust pH to 10-11 (pH paper) with concentrated NaOH. Transfer to separatory funnel.
- 6) Extract alkaline lower phase three times with ether-petroleum hydrocarbon (1:1, by vol.), aspirating upper phase with water line suction.
- 7) Transfer lower phase to round bottomed flask, neutralize to pH 7, and remove ethanol on rotary evaporator.
- 8) Acidify to pH 2 with HCl. Extract twice with ethyl acetate to remove majority of contaminating free acid. Transfer to round bottomed flask.
- 9) Adjust pH to 5 with NaOH. Take to dryness on rotary evaporator.
- 10) Dissolve residue (vigorous shaking) in chloroform-methanol (2:1, by vol.) adding absolute ethanol if necessary to obtain one phase.
- 11) Allow suspension to stand at least 2 hours to insure complete precipitation of all salt. Add Celite (filter aid) and filter through fine mesh filter paper.
- 12) Take clear chloroform-methanol solution of the bile salt to dryness on rotary evaporator.
- 13) Dissolve residue in small volume of water; a clear solution should obtain.
- 14) Pour solution over Dowex 50-W X-2 column (H⁺ form). (Anderson, 1961).
(Dowex has been decolorized previously by repeated cycling with NaOH and HCl.)

Taurocholate and taurodeoxycholate

- a) Extract column effluent as rapidly as possible, twice with ethyl acetate, twice with ether.
- b) Transfer lower phase to round bottomed flask. Neutralize to pH 5 with NaOH. Remove residual ether on rotary evaporator.
- c) Freeze dry:
 - 1) sodium taurodeoxycholate: crystallize from 95% ethanol.
 - 2) sodium taurocholate: no satisfactory crystallization procedure is available. Use the powder obtained by lyophilization.
- d) Dry at room temperature, then at 100° over phosphorus pentoxide and sulfuric acid at less than 5 mm Hg pressure.
- e) Check purity by thin layer chromatography. Apply at least 200 µgm to one spot.

Taurochenoxycholate

- a) Extract column effluent three times with ether.
- b) Proceed as described above in b) through e). No satisfactory crystallization procedure is available. The salt can be precipitated from a methanolic solution by the addition of ethyl acetate.

Flow sheet for synthesis of glycine conjugates of cholic acid, deoxycholic acid, and chenodeoxycholic acid.

Glycocholate and glycodeoxycholate

- 1) Proceed as described on taurine conjugate synthesis flow sheet through step 6).

- 2) Transfer lower phase to round bottomed flask, and remove ether-petroleum hydrocarbon on rotary evaporator.
- 3) Transfer solution to beaker; warm on steam bath. Acidify and triturate with water and ethanol to near turbidity. On cooling, crystallization will occur.
- 4) Recrystallize from ethanol-water. Crystals are to be washed well with water during each filtration step. Air dry crystals after second crystallization.
- 5) Recrystallize crude reaction product from non-aqueous solvent mixtures: glycocholate: dissolve in small volume acetone; add hot ethyl acetate. glycodeoxycholate: dissolve in small volume absolute ethanol; add hot isopropyl ether.
- 6) Check purity of crystals by thin layer chromatography. Crystals should contain no cholic acid and no more than trace amounts of more polar impurities.
- 7) Dissolve crystals in a small volume of absolute ethanol and neutralize to pH 9 with sodium ethoxide. Add ether gradually and sodium salt will crystallize readily.
- 8) Dry as described for taurine conjugates. Recheck purity of preparation by thin layer chromatography.

Glycochenodeoxycholate:

- 1) Proceed as described on taurine conjugate synthesis flow sheet through step 2).
- 2) Dissolve the syrup in water, adding a little NaOH to obtain solution.
- 3) Transfer solution to separatory funnel, acidify (HCl), and extract with ethyl acetate. Wash the ethyl acetate phase repeatedly until water phase is neutral.
- 4) Transfer ethyl acetate phase to round bottomed flask and take to dryness on rotary evaporator.
- 5) Dissolve residue in small volume of methanol and neutralize with NaOH. Remove methanol on rotary evaporator to give a clear, aqueous solution.
- 6) Add 1 M CaCl_2 to solution until precipitation of calcium salt is complete. Filter.
- 7) Crystallize calcium glycochenodeoxycholate twice from ethanol-water (30:70, by vol.).
- 8) Check purity of crystals by thin layer chromatography. A chloroform-methanol solution of the calcium salt can be applied directly to the plate.
- 9) Dissolve crystals in 70% ethanol. Pour solution over Dowex 50-W column (H^+ form).
- 10) Neutralize effluent with NaOH. Take to dryness on rotary evaporator. Dissolve in water and freeze dry. No satisfactory crystallization procedure is available although the salt may be precipitated from a methanolic solution by the addition of ethyl acetate or ether.

Notes:

- 1) The procedures described above for the glycine conjugates eliminate impurities by selective crystallization. An alternate approach for removal of the free acid is liquid-liquid extraction. An aqueous solution of the sodium salt is transferred to a separatory funnel with an equal volume of ethyl acetate. A concentrated solution of KH_2PO_4 is added to the point of slight turbidity.

For glycocholate and glycodeoxycholate, the lower phase is extracted three times with ethyl acetate. The glycine conjugate is crystallized from the lower phase after acidification and trituration with ethanol.

For glycochenodeoxycholate, the extraction is performed with ether-benzene (2:1, by vol.). After further acidification, the glycochenodeoxycholic acid is extracted into ethyl acetate, and the ethyl acetate phase washed well with water. The remainder of the purification procedure is carried out as outlined in steps 4) through 10).

- 2) Occasionally, a more polar impurity (polarity here is defined in terms of thin layer chromatographic mobility) is present in the reaction product. To remove this, a column of silicic acid inactivated with water (Paper II) is prepared in ethyl acetate. The impure glycine conjugate is methylated and an ethyl acetate solution of the methyl ester is poured over column, which is then washed repeatedly with ethyl acetate. The glycine conjugate methyl ester passes through the column; the more polar impurity remains behind. The pooled eluants are evaporated to dryness, and the methyl ester saponified with 1 N NaOH-ethanol (1:1, by vol.) for several hours at room temperature. After removal of the ethanol with a rotary evaporator and acidification, the acid is extracted into ethyl acetate. The remainder of the purification is carried out as outlined in steps 4) through 10).

II. Micellar Aggregation

Hartley (1936), in his brilliant monograph, predicted that bile salts would form micellar solutions. Micellar aggregation in bile salt solutions was subsequently demonstrated by osmotic pressure and freezing point determinations (Roepke and Mason, 1940), by dye solubilization (McBain, Merrill, and Vinograd, 1941; Ekwall, 1951; Sjöblom, 1956; Norman, 1960a; Paper III), by conductivity measurements (Norman, 1960b), by x-ray diffraction (Ekwall and Fontell, 1956; Ekwall, Fontell, and Norman, 1957), by surface tension measurements (Dasher, 1952; Pethica and Schulman, 1952), by cholesterol solubilization (Yoshimuta, 1961), by light scattering (Olson and Herron, 1964), by gel filtration (Borgström, 1964), by electrophoretic mobility (Norman, 1964; Desai and Glover, 1963), and by dye absorption (Hofmann, unpublished observations).

Paper III describes dye solubilization studies with trans-azobenzene, the solute introduced by Hartley (1938). Azobenzene was chosen as a model solute for the determination of the critical micellar concentration (CMC) of each of the individual conjugates and for a comparison of the solvent properties of their solutions. Experimental conditions resembled those present in intestinal content with respect to temperature (37°), pH (6.3), and Na⁺ concentration (.15 M). The dye solubility showed a gradual increase at the CMC and not the abrupt increase observed with typical anionic detergents suggesting that dimers and trimers might form before complete micelle formation occurred. The CMC values obtained were lower than those obtained by Norman (1960a) since they were determined in .15 M Na⁺. In agreement with Norman, the cholate conjugates had a lower CMC than the deoxycholic and chenodeoxycholic conjugates, and there was little difference between the respective glycine and taurine conjugates. Because intestinal content contains a mixture of six bile salts, the CMC of a mixture of bile salts composed to simulate intestinal content was studied. In agreement with the findings of Shinoda (1963) on the CMC of binary and ternary detergent mixtures, the CMC of the mixture was intermediate between the lower CMC of the dihydroxy conjugates and the higher CMC of the trihydroxy conjugates.

The slope of the azobenzene solubility curve, i.e. the ratio of moles of micellar solute to moles of micellar solvent, was defined as

as the saturation ratio. The saturation ratio of azobenzene in bile salt solutions was considerably below that of azobenzene in typical anionic detergent solutions, unless a polar additive such as monoglyceride was cosolubilized.

Since the predominant micellar solutes in intestinal content are polar lipids, the solubilization of a model polar lipid, glycerol 1-monooleate, was studied. The CMC observed with this polar solute was far below that observed with azobenzene, in agreement with Lawrence and Stenson (1957) and Shinoda (1963) who showed that polar additives caused a marked lowering of the CMC. Polar lipids such as monoglycerides may be termed amphiphiles, i.e. water insoluble amphipaths which under appropriate conditions show high micellar solubilities in detergent solutions and when present in excess form a liquid crystalline phase with the detergent and water.

Temperature effects:

Most ionic detergents show a remarkable increase in solubility over a very narrow temperature range (Krafft point). At any temperature, a detergent has a limited but significant molecular solubility. If an excess is present, it will be crystalline or micellar depending on the experimental temperature. Shinoda and Soda (1963) have presented evidence based on partial molal volume data that the Krafft point represents the melting point of the hydrated ion (or ion pair) and corresponds to a phase change. They showed that a change in partial molal volume of a soap occurred at the Krafft point irrespective of whether the soap was above or below its CMC. The Krafft point may be termed a critical micellar temperature (CMT) (Hofmann, 1964b). Thus for a detergent to exist in micellar solution, it must be above its CMC and CMT.

Whether bile salts possess Krafft points has not been considered previously. The Krafft point of the sodium salts of the common bile salt conjugates is below 0°. Although sodium lithocholate (3 α hydroxy substituent) is generally stated to be insoluble in water, micellar solutions occur above 60°. With sodium cholanate (no hydroxy substituents), the Krafft point is above 100°. These observations suggest that the Krafft point

falls with increasing number of hydroxy substituents. (For observations on the Krafft point of stearate and 9, 10 dihydroxy-stearate, see Gregory and Tartar, 1948). Information is needed on the influence of number, position, and orientation of substituents on the Krafft point of bile salts.

The Krafft point of any anionic detergent is influenced greatly by the nature of the counter ion present. Although calcium salts of carboxylate soaps are insoluble in water, their insolubility merely reflects their possessing a Krafft point greater than 100° . The Krafft point of sodium dodecyl sulfate is about 20° ; that of calcium dodecyl sulfate is about 55° . It should be possible to plot the Krafft point of a detergent at a fixed concentration with varying molar ratios of sodium to calcium ions, just as Raison (1957) has plotted the Krafft point of varying molar ratios of dodecyl sulfate and tetradecyl sulfate at a fixed sodium ion concentration. This has not been done. The Krafft point of sodium lithocholate is about 60° ; that of tetramethyl ammonium lithocholate is below 30° .

The Krafft point of calcium glycodeoxycholate is above 100° , and the Krafft point of other calcium salts of bile salt conjugates has not been examined. The Krafft points of glycodeoxycholate with differing ratios of calcium and sodium ions has received attention because of the occurrence of gallstones composed of crystals of a sodium and calcium salt of the 5α isomer of glycodeoxycholic acid in the cholestanol fed rabbit (Hofmann and Mosbach, 1964). If calcium ions are added to a solution of sodium glycoallodeoxycholate (the glycine conjugate of the 5α isomer of deoxycholic acid), the insoluble calcium salt precipitates at a far lower calcium concentration than is observed with an identical solution of glycodeoxycholate, the 5β isomer. This indicates that the Krafft point of the 5α isomer is higher for a given ratio of calcium to sodium ions.

Although the Krafft points of sodium tauroallodeoxycholate (5α) and taurodeoxycholate (5β) are both below 0° , one can demonstrate a striking difference between the two bile salts, if the Krafft point of a bile salt-palmitate mixture at a 1:1 micellar ratio is determined. The

Krafft point of sodium tauroalloodeoxycholate (5 α)-palmitate is 75°; of taurodeoxycholate (5 β)-palmitate, 32° (Hofmann, unpublished observations).

To summarize, the low Krafft point of bile salts appears to be related to at least two factors: the number (and possibly arrangement and orientation) of hydroxy groups; and the type of A/B ring fusion.

Azobenzene solubility in micellar bile salt solutions was higher at 37° than 25°; the difference observed was similar to that reported for azobenzene solubilization at 25° and 37° in a number of anionic detergents.

The effect of high temperature (>80°) on micelle formation in bile salt solutions has not been examined. Such studies might enable an assessment of the importance of hydrogen bonding in micellar aggregation.

Phase equilibria:

Binary systems of soap and water have been studied by many workers (see, for example, McBain and Lee, 1943). The conjugated bile salt-water system has no liquid crystal phase (Paper IV). A liquid crystalline phase is seen with 5 β bile salts at high salt concentration; with 5 α bile salts, however, a liquid crystalline phase is seen at relatively low bile salt concentrations even in the absence of electrolyte. Presumably the flat molecule of the 5 α conjugate packs more readily into a bimolecular leaflet, although no information is available on the type of molecular packing in any of these liquid crystalline phases.

Micellar size and arrangement:

Until very recently, aside from a single report (Moerlose and Ruyssen, 1959), the techniques of light scattering and ultracentrifugation had not been applied to the determination of the micellar size in bile salt solutions. Olson and Herron (1964) have recently reported micellar weights of 11,000 and 3,000 for sodium taurodeoxycholate and sodium glycocholate respectively; and using gel filtration, Borgström (1964) has estimated the micellar diameter to be about 40Å for a bile salt micelle containing an amphipathic additive. The bile salt micelle occurring in bile and intestinal content contains six different types of bile salt molecules and both polar and non-polar lipid solutes. It seems probable that the micellar weight of this micelle (in the presence of physiological concentrations of electrolytes) will be in the range of 10,000 - 30,000,

a size comparable to the micellar weights of many short chain anionic detergents in water.

There is no information on the molecular arrangement of the bile salt micelle. The bile salt molecules can be considered to have a hydrophobic top and a hydrophilic bottom, in contrast to most anionic detergents which have a charged head on a long hydrophobic body. One might term bile salts planar amphipaths and anionic detergents polar amphipaths. Other planar amphipaths are hydroxy fatty acid soaps and probably certain dye molecules which aggregate in solution (Alexander and Stacey, 1952). It seems unlikely that the bile salt micelle is spherical, and the only reasonable alternatives would seem to be a lamellar or helical arrangement. Optical rotatory dispersion measurements in the far ultraviolet would be of interest.

Much more information is needed about the influence of position and orientation of hydroxy or other substituents on micellar aggregation. Ekwall (1951) showed that the triketo derivative of cholic acid (as the sodium salt) does not form micelles. The 3α , 7α , dihydroxy-12 keto-derivative of cholic acid (as the sodium salt) has a far higher CMC than sodium cholate (Hofmann, unpublished observations). Ultimately, it should be possible to construct a model of the micelle using projections of Dreiding models, as so beautifully done for the myelin sheath by Vandenheuvel (1963).

pH:

Approximate pK_a 's for several of the glycine and taurine conjugates have been measured (for citations, see Ekwall, Rosendahl, and Löffman, 1957). The pK_a ' of any detergent will be considerably higher when its concentration is above the CMC (Veis and Hoerr, 1960; Hartley and Roe, 1940), and many workers have neglected this consideration (but see Ekwall, Rosendahl, and Löffman, 1957). Measurement of the pK_a ' of the glycine conjugates is also complicated by the insolubility of the unionized acid, but on the basis of the values reported for glycine and taurine conjugates, it is probable that the carboxyl and sulfonate groups of the glycine and taurine conjugates are almost completely ionized at the pH present in bile and in intestinal content.

Azobenzene solubilization by bile salt solutions was not influenced by pH (Paper III). Important pH effects are observed, however, when the micellar solubility of ionizing solutes such as fatty acid is studied.

Binding of counterions:

Micelles can be considered as polyelectrolytes, and all polyelectrolytes bind counter ions; the binding can be treated mathematically by the same equations used for ion exchange equilibria. Since the development of cation specific electrodes, the binding of counter ions by detergent micelles has received renewed attention (Shedlovsky, Jakob, and Epstein, 1963) and several models have been proposed (e.g. Clifford and Pethica, 1963). Shinoda (1963) has tabulated results reported for micellar charge based on light scattering, electrophoretic mobility measurements, and osmotic coefficient determinations. Recently, Pearson and Lawrence (unpublished observations) have shown that the soap micelle binds sodium ions but will release these if either calcium ions or an amphiphile is added.

The bile salt micelle also binds sodium ions (Moore and Dietschy, 1964), but no studies have been performed on the binding of calcium ions by the bile salt micelle, nor has the effect of amphiphilic additives on counter ion binding been investigated. Some physiological implications of cation binding by bile salts have been discussed (Hofmann, 1964b).

Solutions of the sodium salts of the normally occurring 5β conjugates are far more resistant to precipitation by the addition of calcium ions than solutions of typical anionic detergents or 5α conjugates. As noted, this reflects the lower Krafft point of the 5β isomer with a given ratio of sodium and calcium ions. Since the Krafft point of calcium glycodeoxycholate $[Ca(GDC)_2]$ is above 100° , one must be able to explain the anomalously high solubility of bile salt in the presence of the appreciable calcium ion concentration in bile and intestinal content. Therefore, the following solubility product relationship was proposed (Hofmann and Mosbach, 1964).

$$K_{sp} \text{ of } Ca(GDC)_2 = [Ca^{++}] [GDC^-]^2$$

$$\text{but } [Ca^{++}] = \text{total } [Ca^{++}] - [Ca^{++}]_{\text{bound to the micelle as counter ion}}$$

$$\text{and } [GDC^-] = \text{total } [GDC^-] - [GDC^-]_{\text{in micellar form}} = \text{CMC of } GDC^-$$

$$\text{Hence } K_{sp} \text{ of } Ca(GDC)_2 = [Ca^{++}]_{\text{unassociated}} [CMC \text{ of } GDC^-]^2$$

Hence the failure of calcium glycodeoxycholate to precipitate from bile indicates that the product of unbound calcium ions and the CMC squared is less than the true solubility product. (The above discussion neglects activity coefficients.) One might regard the mixture of bile salts occurring in bile as analogous to the sulfonamide mixtures which were designed to take advantage of a common therapeutic activity despite independent solubility products (Lehr, 1948).

Calcium ion:

The formation of insoluble calcium soaps would seem as undesirable in the intestinal lumen or gallbladder as it is in the dishpan or bathtub. The striking resistance of 5 β bile salts to the addition of Ca⁺⁺ ions has been noted and explained, and the importance of the type of A/B ring junction has been stressed. It would be of interest to compare the effect of adding calcium ions to solutions of sodium oleate (cis) and sodium elaidate (trans), these soaps being analogous stereoisomers.

III. Behavior of solutes of physiological importance in micellar bile salt solutions.

In considering the behavior of any lipid as a micellar solute, it is helpful to distinguish polar and non-polar solubilization which have been defined, at least empirically (Lawrence, 1961). It is convenient to use the term amphiphile (p.10) for compounds behaving as polar solutes under appropriate conditions. Whether a lipid behaves as a polar or non-polar solute is influenced by temperature, by the type of detergent, and indeed by poorly understood molecular interactions between the solute and the detergent molecules in which steric factors are undoubtedly of great importance. Whenever there is interaction between solute and micellar solvent, the solubility relationships can be expressed completely only by phase diagrams. Two types of phase diagrams are required: the first, a ternary composition phase diagram at constant temperature; the second, a diagram with rectangular coordinates in which the phase changes with varying amphiphile/detergent ratios are plotted against temperature (Lawrence, Hume, Capper and Bingham, 1964). These two phase diagrams can be united

in a prism (Findlay, 1923) in which the triangles representing the ternary composition phase diagram at each of a number of temperatures are mounted above each other.

Triglycerides and diglycerides:

Triglycerides and diglycerides, irrespective of m.p., behave as non-polar solutes in bile salt solutions. Their micellar solubilities are low, and the excess is crystalline or emulsified depending on the m.p. of the solute and the experimental temperature. Eutectic points are not observed. The influence of chain length on diglyceride and triglyceride solubility in bile salt solution has not been investigated.

As noted, the solubility of certain non-polar solutes is increased greatly by the addition of polar solutes to the micelle. There has been no systematic study of the effect of added amphiphile on diglyceride or triglyceride solubility in bile salt solution. Small amounts of diglyceride and triglyceride are present in the micellar phase isolated from human intestinal content (Paper VI) indicating that these lipids possess measurable solubilities in the mixed micelle present in intestinal content, even if their solubilities in bile salt solutions alone are very small. In vitro, the solubility of glyceryl dioleate in bile salt solution is increased by the addition of glyceryl 1-monooleate (Hofmann, unpublished observations). Burdett and Pover (1963) have recently reported that the addition of fatty acid and monoglyceride increases the solubility of triglyceride in bile.

To summarize, the limited data available suggest that the solubility of diglyceride and triglyceride in bile salt solution increases somewhat with the addition of polar solutes, but the effects observed are of small magnitude; diglyceride and triglyceride probably do not occur as micellar solutes to any appreciable extent in vivo.

Monoglycerides:

The behavior and solubility of monoglycerides in buffer and in bile salt solution was reported in Paper IV. This paper noted the low solubility of long chain monoglycerides in water, but failed to report that glyceryl 1-monolaurate forms a liquid crystalline phase in water at 44°. It is now clear that all saturated straight chain monoglycerides

of chain length $\geq C_{12}$ form a liquid crystalline phase in water at appropriate temperatures (Lawrence et al., 1964). Thus monoglyceride is a non-ionic analogue of lecithin, in terms of its behavior in water. Lawrence has suggested that monoglyceride and phospholipid are members of a large class of compounds occurring in nature which always remain associated in water, and that it is helpful to say that water is soluble in them, even if they are insoluble in water (Lawrence et al., 1964).

The monoglyceride-water liquid crystalline phase occurs at temperatures well below the m.p. of the anhydrous monoglyceride. This eutectic point may be determined easily by observing the temperature at which penetration of crystals of monoglyceride suspended in water occurs, when the suspension is heated slowly on a microscopic stage. Lawrence et al. (1964) have recently reported this penetration temperature (T_{pen}) for a number of straight chain, saturated monoglycerides in water. The temperature difference observed between the m.p. of the anhydrous compound and its T_{pen} ($m.p. - T_{pen}$) is abbreviated Δ_T . For the straight chain saturated monoglycerides from C_{12} to C_{18} , the Δ_T is 17-27°, irrespective of the crystal form of monoglyceride used.

Paper IV showed that at 37° monoglycerides of low m.p. had remarkably high micellar solubilities in bile salt solutions, but that monoglycerides of high m.p., such as glyceryl 1-monopalmitate behaved as non-polar solutes. If composition and temperature phase diagrams had been determined for each of the homologous monoglycerides, it would have been evident that glyceryl 1-monopalmitate possesses high micellar solubilities at higher experimental temperatures, and thus that whether it behaves as a non-polar solute or polar solute is determined solely by the experimental temperature. Fig. 2 shows a phase diagram in which varying ratios of glyceryl 1-monomyristate and dilute sodium taurodeoxycholate are plotted against temperature. Of interest is 1) the presence of a eutectic (the liquid crystalline phase occurs some 25° below the m.p. of the anhydrous monoglyceride) and 2) the striking increase in the micellar area over a narrow temperature range. The temperature at which this striking increase in monoglyceride solubility occurs may be considered as a Krafft point for the ternary system.

Lawrence has recently constructed the ternary composition phase diagram for glyceryl 1-monolaurate-sodium taurodeoxycholate-water (Fig. 1). The solubility figures reported in Paper IV represent the boundary of the isotropic region in this phase diagram, which differs from most soap-water-amphiphile phase diagrams by its enormous isotropic regions. A lauryl sulfate-glyceryl 1-monolaurate-water phase diagram would probably show a much larger liquid crystalline and a correspondingly smaller isotropic area (c.f. Paper III). Composition and temperature phase diagrams are needed for each of the homologous saturated straight chain monoglycerides as well as the more common unsaturated monoglycerides. Whether it will be worthwhile to construct phase diagrams with each monoglyceride and each bile salt conjugate is difficult to predict.

Because of the unavailability and instability of the 2-monoglyceride isomers, only limited studies of the behavior of these physiologically important isomers were carried out. No differences were noted in the micellar solubility of 1- and 2-monoolein, and the differences observed between 1- and 2-monopalmitin could have been entirely due to differences in their m.p. Since the glyceryl ethers appear to have similar physical properties, the 1- and 2-glyceryl ethers with homologous saturated and unsaturated alkyl chains should be prepared and compared with respect to their behavior in water and bile salt solutions.

The problem of competitive solubilization, which has received but brief attention in the literature (Ekwall, Sten, and Norman, 1956; Matuura, Furudate, Tsutsumi, and Miida, 1960), was studied (Paper IV). Such a problem is of considerable physiological interest because of the variety of monoglycerides which are present in intestinal content during fat digestion. However, interpretation of the experiments in paper IV is impossible because of our ignorance of the molecular arrangement of the bile salt micelle.

When ranked in order of solubility in bile salt solution, dodecanol < glycol 1-dodecanoate < glyceryl 1-dodecanoate. These observations suggest that the number and position of hydroxy groups is important in determining solute-bile salt interaction; hydrogen bonding is probably

involved. Temperature and composition phase diagrams are needed for the 1- and 2-monoether analogues, the analogues listed, as well as the erythritol 1-monoester, etc. Penetration experiments (Schulman and Friend, 1949) might give further insight into bile salt-amphiphile interaction. In such experiments, bile salt would be injected into the solution below a monolayer of amphiphile, and the changes in surface pressure recorded.

To summarize, all long chain monoglycerides possess high micellar solubilities in bile salt solution at temperatures greater than 17-27° below the m.p. of the anhydrous monoglyceride. The β fatty acids of virtually all natural triglycerides are predominantly unsaturated (Coleman, 1963), and since β monoglyceride is formed by the selective action of pancreatic lipase, the monoglyceride normally formed during fat digestion should possess high micellar solubility. Lard appears to be unique in having a relatively high percentage of saturated fatty acids in the β position, but it remains likely that the mixture of β monoglycerides formed by pancreatic lipolysis of lard has a T_{pen} below 37° and a high micellar solubility.

In model systems of bile salt, monoglyceride, and water, excess monoglyceride forms a liquid crystalline phase or a crystalline suspension. In intestinal content, however, an emulsified oil phase composed of higher glycerides and fatty acid coexists with the micellar phase (Paper VI), and the concentration of monoglyceride present in the micellar phase must reflect the partition coefficient of the monoglyceride between these two phases. Experiments cited in paper VI indicate that glyceryl 1-monooleate has a partition coefficient of 0.1 when partitioned between triolein and sodium taurodeoxycholate.

$$K = \frac{[\text{glyceryl 1-monooleate}]/\text{moles triolein}}{[\text{glyceryl 1-monooleate}]/\text{moles } \underline{\text{micellar}} \text{ sodium taurodeoxycholate}} = 0.1$$

For monoglycerides of higher m.p., it is probable that the partition coefficient will fall when the micellar solubility increases greatly, i.e. at temperatures $\geq T_{pen}$.

Fatty acid:

The predominant lipid in the micellar phase isolated from human intestinal content is fatty acid (Paper VI). Understanding of bile salt-fatty acid interaction is essential if fat digestion and absorption are to be explained using conventional concepts of physical chemistry. This discouragingly complex problem is under study (Hofmann, 1965), and recent experiments are summarized here, which although incomplete, define the problem more clearly. The term soap is used in this section to denote the fatty acid anion.

Since monoglyceride does not ionize, there is no effect of pH on the monoglyceride-bile salt-water composition phase diagram. Fatty acids can ionize to soaps under appropriate conditions, and titration experiments indicate that the fatty acid present as micellar solute in bile salt solution has a pK_a (the bulk pH at which the fatty acid is half ionized) of about 6.5, and that this pK_a is not influenced by more than 0.5 pK_a units by temperature, type of bile salt, micellar fatty acid to micellar bile salt ratio, or the presence of added monoglyceride (Paper IV; Hofmann, 1965). Since the pH of intestinal content is 6.0-6.5, the micellar fatty acid present should be partially ionized. A schematic representation of the titration of micellar fatty acid is shown in Fig. 5.

As indicated previously, monoglyceride-bile salt interaction can be adequately described only by constructing a composition and temperature phase diagram for each monoglyceride; this is also true for fatty acid-soap-bile salt interaction. One needs to construct the composition and temperature phase diagrams of the bile salt-fatty acid-(soap)-water system (1) at acid pH, when the fatty acid is completely undissociated (such a diagram can only be constructed with the taurine conjugates) (2) at alkaline pH, when the fatty acid is fully ionized, i.e. present as soap, and (3) at intermediate pH, e.g. pH 6.5, when the fatty acid is half ionized. Obviously, the construction of such phase diagrams for a series of homologous fatty acids is a very laborious undertaking.

Further, any description of the behavior of fatty acid or soap in bile salt solution should be compared to the behavior of fatty acid and

soap in water (or buffer). This problem is poorly understood despite considerable experimental work (Stainsby and Alexander, 1949; Goddard and Ackilli, 1963). The intrinsic pK_a of any long chain fatty acid must be about 4.8, but in aqueous solution, with fatty acids of chain length greater than C_{10} , insolubility and molecular association complicate the titration curves (see, for example, Schmidt-Nielsen, 1946). Fatty acids behave in water as if their pK_a were far higher than 4.8.

Consider the titration (with acid) of an isotropic solution of a paraffin chain soap at concentrations above and below its CMC. With a soap above its CMC (and by definition at a temperature above its Krafft point), the association of a micellar fatty acid anion with a proton can be treated with conventional polyelectrolyte equations, since each anion is surrounded by a number of neighboring anions. After partial neutralization, there is a phase change with separation of a solid (acid soap) or liquid crystalline phase. The ionized carboxylate groups are now in a different spatial arrangement; moreover, the charge density is diminishing continuously with neutralization. A biphasic titration curve for sodium laurate has been reported (Schulman, Stoeckenius, and Prince, 1959) in which the first point of inflexion signified the separation of an acid soap phase and the second, the separation of the unionized acid. With titration of a soap below its CMC (and neglecting temperature effects) there is the initial formation of dimers (Franks, personal communication) and again interaction between ionized and unionized groups. The separation of unionized free acid from solution, which occurs at relatively high pH, is a phase change and can be considered to increase markedly the pK_a since the crystalline free fatty acid acts as a sink for the protons added to the solution and does not participate in the equilibria expressed in the Henderson-Hasselbach equation (Schmidt-Nielsen, 1946). Despite these complexities, however, in buffers of pH corresponding to that present in intestinal content, all long chain fatty acids exist solely in the form of the unionized fatty acid and, as such, have negligible solubilities.

The behavior of homologous saturated fatty acids in dilute sodium taurodeoxycholate has recently been investigated (Hofmann, 1965). At acid pH, in buffer alone, fatty acids possess negligible solubilities. In bile salt solution, fatty acids behave as non-polar solutes. The excess is either crystalline or emulsified; liquid crystalline phases have not been observed. The T_{pen} of fatty acid in bile salt solution has been measured; the ΔT is not more than two degrees and does not differ significantly from ΔT in water alone (Lawrence, personal communication). At experimental temperatures below the m.p. of a given fatty acid, micellar solubilities are low, although considerably above that of azobenzene. When the fatty acid is liquid, its micellar solubility rises markedly. These relationships are shown in Fig. 3.

Since the T_{pen} of fatty acid in bile salt solution is little different than its m.p., a fatty acid differs from its homologous monoglyceride in requiring a higher temperature to manifest significant micellar solubility, despite its anhydrous m.p. being lower.

Older work on the behavior and solubility of fatty acid in bile salt solution (Holwerda, 1937; Breusch, 1937) is difficult to interpret. Verkade and Meerburg (1955) studied the solubility of homologous saturated fatty acid in sodium glycocholate solutions, and although pH effects were neglected, these workers demonstrated clearly that the solubility of fatty acid is greater with decreasing chain length and with a given fatty acid, increases at experimental temperatures above the m.p. of the fatty acid.

It might be mentioned parenthetically but in contrast, that in solutions of typical ionic detergents, at acid pH, fatty acids have moderate micellar solubilities, have eutectics, and form a liquid crystalline phase in excess. The system detergent-fatty acid-water has been studied by several laboratories that chose fatty acid as a representative amphiphile. Lawrence (1961) has reported the T_{pen} and ΔT of fatty acids in various detergent solutions.

To summarize, the solubility of fatty acid in bile salt solution with experimental conditions such that no fatty acid ionization occurs is appreciable only above the m.p. of the fatty acid and even then, the

saturation ratios obtained are below those obtained for a homologous monoglyceride. Further, since no eutectic is observed, the temperature required for appreciable micellar solubility is close to that of the m.p. of the anhydrous fatty acid. Therefore at 37° the solubility of all long chain saturated fatty acids above C₁₄ is quite low. Even with the unsaturated fatty acids of low m.p., such as oleic acid, saturation ratios at 37° are much below those of the homologous monoglyceride. Therefore, the high micellar solubility of fatty acid observed in vivo cannot be explained satisfactorily by interaction between the undissociated fatty acid and the bile salt micelle.

At alkaline pH, soap has negligible solubility if below its Krafft point or a very high solubility (limited by the appearance of the liquid crystalline phase) if above its Krafft point. Complete phase diagrams have been constructed for soap-water systems (McBain and Lee, 1943). Bile salts, as noted, have a Krafft point well below 0°, and it is possible to construct a phase diagram in which the Krafft point of bile salt-soap mixtures of varying micellar molar ratios are plotted, just as Raison (1957) has plotted the Krafft points of mixtures of homologous alkyl sulfates. Such a diagram indicates that bile salts act to increase greatly the solubility of any soap at a given temperature by acting as a detergent with a Krafft point which extrapolates to far below 0° (Fig. 4). A similar curve should be obtained if the Krafft point of a mixture of varying ratios of a highly branched ionic detergent (such as Teepol) and a soap was plotted. These findings suggest that the high micellar solubilities of fatty acid in bile salt solution observed in vivo can be explained in terms of soap-bile salt interaction, or bile salt-soap-fatty acid interaction.

The problem at intermediate pH, such as is present physiologically, is extremely complicated because pH and temperature are now interdependent. What appears to be required is the construction of a prism, i.e. a composition phase diagram for a number of temperatures at each pH. Further, one requires a series of prisms for each fatty acid. Experimentally, if one

warms a suspension of myristic acid in dilute sodium phosphate buffer (pH 6.5, for example) and taurodeoxycholate at a concentration several times its CMC, the pH of the solution falls. The fatty acid melts, enters the micelle, ionizes, and liberates protons which exchange with sodium counter ions; the pH falls. With the fatty acid-soap combination of saturated fatty acid existing at intermediate pH the solubility can be increased either by raising pH or increasing temperature; with unsaturated fatty acid, solubility can be increased only by raising pH.

Hartley, struck by the change in pK_a of indicators when they were solubilized in micellar solutions defined and elegantly studied the "surface pH" of a soap micelle (Hartley and Rose, 1940). The state of ionization of a fatty acid present as micellar solute is another example of the same problem. The pK_a of a micellar fatty acid may vary from 5.2 when in a cationic detergent micelle to 7.2 when in an alkyl sulfate micelle (Hofmann, 1965). The pK_a of a fatty acid dissolved in a bile salt micelle is about 6.5, which is 0.6-0.8 pK_a units below that of a fatty acid dissolved in a typical anionic detergent micelle (Fig. 6). If one defines the pK_a of a fatty acid in an aqueous solution as the bulk pH at which the fatty acid is half ionized, the pK_a of a fatty acid in water is probably above 8; in an anionic detergent solution, 7.0-7.4; and in a bile salt solution, 6.4-6.9. The solubilization of fatty acid in the bile salt micelle enhances fatty acid ionization relative to fatty acid ionization in water or other anionic detergent micelles.

This discussion has considered bile salt-fatty acid-soap interaction and neglected consideration of 2-monoglyceride, the other major component of the micelle occurring in vivo. This problem can be studied experimentally by constructing phase diagrams at acid and alkaline pH using glyceryl 2-monoethers as the stable analogues of 2-monoglycerides. At acid pH and 37°, added monoglyceride (either unsaturated or of low m.p.) will increase the micellar solubility of high m.p. fatty acid. This phenomenon is not dissimilar to the increase in azobenzene solubility in bile salt solutions when monoglyceride is added. However, frequently

the combination of monoglyceride and fatty acid behaves as a polar solute, having a eutectic, high micellar solubility, and the formation of a liquid crystalline phase when excess is added. Thus, at acid pH, the addition of amphiphile increases the solubility of fatty acid and thus has the same effect as an increase in experimental temperature.

The effect of added amphiphile on soap-bile salt interaction at alkaline pH has not been studied. At intermediate pH, added amphiphile increases the solubility of fatty acid (and soap) of fatty acid of high m.p. With fatty acids of low m.p., the solubilization becomes competitive (Hofmann and Borgström, 1962).

Considering the above, it is possible to make a cautious statement of the factors responsible, at least in part, for the high solubility of fatty acid-soap in bile salt solutions: (1) the low micellar solubility of saturated fatty acid below its m.p. is increased by the presence of unsaturated monoglyceride; the monoglycerides resulting from the digestion of natural fat are largely unsaturated (2) the ionization of fatty acid to soap is enhanced by solubilization in the bile salt micelle, this factor is largely responsible for the high micellar solubilities of unsaturated fatty acid at intermediate pH; and (3) the bile salt micelle can readily incorporate soap of high Krafft point at 37°, because the Krafft point of the bile salt micelle is so low. To summarize, the proposed scheme thus provides for high fatty acid solubility at acid pH and high soap solubility at alkaline pH. Assuming that some combination of these results in high fatty acid and soap solubility at intermediate pH, fatty acid-soap solubilization becomes almost independent of pH, and an explanation is available for the observation that fat digestion and absorption proceeds normally despite considerable variation in intraluminal pH. However, it seems probable that alkalinization of intestinal content by pancreatic juice bicarbonate is an essential part of fat digestion. For appreciable micellar solubilities of an unsaturated fatty acid-soap, e.g. oleic, are not obtained unless the pH is high enough for significant ionization of solubilized fatty acid.

Although the property of the bile salt micelle to enhance fatty acid ionization appears to be responsible for the high micellar solubility of unsaturated fatty acid at intestinal pH, this enhancement of ionization also has physiological implications: (1) when a fatty acid is ionized in the bile salt micelle, the fatty acid anion can no longer take part in transesterification catalyzed by pancreatic lipase; hence, pancreatic lipolysis is driven in the direction of more complete hydrolysis; (2) as triglyceride is hydrolyzed to fatty acid, which is then solubilized, and ionizes, protons are liberated; pancreatic juice bicarbonate thus serves to neutralize fatty acids as well as gastric juice hydrochloric acid; and (3) the bile salt micelle has counter ions associated not only with its own anionic groups but also with the anionic groups of the ionized fatty acids.

As noted, an emulsified oil phase of higher glycerides and fatty acid is believed to coexist with the micellar phase of bile salt, fatty acid, soap, and monoglyceride (Paper VI). The partition coefficient of fatty acid between model micellar and oil phases at varying pH should be studied.

Lastly, it seems unlikely that any anionic detergent exists whose micellar solutions possess as high a saturation ratio for the mixture of fatty acid, soap, and monoglyceride occurring in vivo. The striking micellar solubility observed reflects a remarkable combination of four classes of surface active molecules: fatty acid, monoglyceride, soap, and bile salt. Their hydrophile-lipophile balance increases in the order listed.

Cholesterol:

Cholesterol behaves as a non-polar solute in bile salt solutions. Its micellar solubility is low, but is greatly increased as the bile salt micelle is swollen with amphiphile such as monoglyceride or lecithin (Yoshimuta, 1961; Isakson, 1953; Hofmann and Borgström, 1962; Spanner and Bauman, 1932). The temperature and composition phase diagrams for the lecithin-cholesterol-bile salt solution system need delineation because of their bearing on cholelithiasis.

Micellar Substrates:

Water insoluble substrates can be dispersed in water in appreciable concentration in four types of aggregates: micelles, liquid crystalline fragments, emulsions, or suspensions. The use of emulsified substrates is common, and liquid crystalline substrates have recently received attention (for references, see Bangham, 1963) and their value has been demonstrated. Paper V presents experiments in which micellar solutes were used as substrates for pancreatic lipase.

The experiments demonstrated directly for the first time the positional specificity of pancreatic lipase and also described an extremely stable and easily prepared substrate for pancreatic lipase which may prove of value in clinical laboratories. The use of micellar substrates prepared with other detergents, for example, non-ionic detergents, would seem worthy of investigation. It is improbable that appreciable hydrolysis of micellar substrates occurs in intestinal content, except when large amounts of 1-monoglycerides are fed.

Interpretation of the experiments in paper V will be aided by elucidation of the molecular arrangement of the bile salt-monoglyceride micelle.

IV. Isolation of a micellar phase from intestinal content during fat digestion and absorption.

The problem of fat digestion and absorption will be dealt with but briefly, as three thorough and very readable reviews have recently been published (Johnston, 1963; Clement, 1964; Senior, 1964). Each contains an extensive bibliography, which includes all of the citations omitted below.

Physical state:

The idea that lipids might be dispersed in a form other than emulsion droplets during fat absorption is very old (Tschernoff, 1884; Moore and Rockwood, 1897; Moore and Parker, 1901) and long preceded any knowledge of the physical chemistry of detergent solutions. Verzar in ignorance of the then emerging concepts of micelle formation, nonetheless realized that

the choleic acid concept of Wieland and Sorge was incorrect and actually drew pictures of polymolecular aggregates of bile salt and fatty acid. McBain proved that bile salts were detergents and formed micellar solutions but was apparently unaware of pancreatic lipolysis. Frazer, in collaboration with Schulman, demonstrated the powerful emulsifying properties of the combination of bile salt, monostearate, and oleic acid for triglyceride. He subsequently isolated monoglycerides from intestinal content during fat absorption and used their existence as evidence that his in vitro emulsifying system was present in vivo. Dowse, Saunders, and Schofield (1956) and Borgström (1960) separated intestinal content into an emulsified oil phase and a subnatant phase which contained lipid in a more finely dispersed form.

Chemical form:

Since Claude Bernard's demonstration that pancreatic juice splits triglyceride to fatty acid, it has been generally held that ingested fat is absorbed largely as fatty acid (or soap). Frazer dissented saying that (1) lipolysis was incomplete, and partial glycerides with important emulsifying properties were present during absorption and absorbed as such and (2) considerable triglyceride was absorbed without any lipolysis. With the establishment of the positional specificity of pancreatic lipase, Frazer's demonstration that partial glycerides were present in intestinal content received new emphasis. It was probably Reiser (1955) who first clearly proposed that fat was absorbed in the chemical form of monoglyceride and fatty acid. This proposal was in accord with the then recent findings of Bernhard, Wagner, and Ritzel that the label of C¹⁴-glycerol labeled triglyceride was retained in chylomicron triglyceride. A few years later, Skipski, Moorhouse, and Deuel (1959) presented evidence for the intact absorption of monoglyceride.

Therefore paper VI is a logical extension of these investigations. The proposed scheme of fat absorption in paper VI unites harmoniously many of the ideas of Verzar and Frazer and it is clear in retrospect that the lipolytic and particulate hypotheses were more incomplete than incorrect.

The necessary conceptual foundation for describing the process of micellar solubilization was provided by Lawrence's incisive experiments with soap-water-amphiphile systems. Both Verzar and Frazer sought to correlate in vitro observations on model systems with studies in the whole animal, and the work in this dissertation has done this also.

The recent demonstrations (Saunders and Dawson, 1962; Holt, 1963) that under appropriate conditions free glycerol is incorporated into chylomicron triglyceride has made impossible a quantitative assessment of the extent of lipolysis during fat absorption. However, the agreement of numerous laboratories that the β fatty acid of ingested triglyceride is not randomized during lipolysis and mucosal reesterification is convincing evidence that lipolysis is not complete and strongly suggests that absorption is in the form of 2-monoglyceride and fatty acid.

Paper VI thus proposes that the bile salt micelle may well be the "final common path" of all water insoluble materials which are absorbed from the intestine. Borgström made the attractive suggestion that the partition coefficient of lipids between the micellar and oil phases might partly explain the puzzling problem of the selectivity of lipid absorption.

Perfusion experiments (Paper VII) showed that fatty acid and monoglyceride when dissolved in a bile salt solution were readily absorbed from the human small intestine. These experiments have recently been confirmed by Kayden (personal communication) who injected a micellar solution of doubly labeled 2-monoglyceride, fatty acid, and bile salt into the intestinal lumen of a subject with a thoracic duct fistula. The lipid was readily absorbed and appeared as chylomicron triglyceride. The distribution of radioactivity in the isolated triglyceride indicated that the 2-monoglyceride had been absorbed and acylated without hydrolysis. These experiments confirm our demonstration that micellar fatty acid and monoglyceride can be absorbed and acylated in a way similar to that proposed for ingested triglyceride (Johnston, 1963; Senior, 1964). Johnston and Borgström (1964) have recently published a thorough study on the uptake and acylation of micellar fatty acid and monoglyceride by everted hamster gut sacs.

The intraluminal phase of triglyceride absorption now comprises at least four steps (1) emulsification (2) enzymatic lipolysis (3) micellar solubilization and (4) passage of the micelle and/or its solutes into the mucosal cell. Although it is possible that the concepts presented here with respect to (3) will help the interpretation of the numerous experiments of past decades on the efficiency of absorption of various natural and synthetic triglycerides, it is more likely they will prove inadequate. For example with synthetic triglycerides of high m.p., (1), (2), and (3) probably do not proceed normally.

Paper VI leaves many questions unanswered. Some questions, such as the distribution of phospholipids between the oil and micellar phases, was not examined. Lipid analyses were by class only. The distribution of such important solutes as cholesterol, cholesterol esters, and fat soluble vitamins was not examined.

V. The intestinal site of bile salt absorption.

Previous work in animals by Lack and Weiner (1961) and Baker and Searle (1960) as well as the older work of Fröhlicher (1935) had indicated that bile salt absorption occurred in the distal small intestine. In contrast, the classical observations of Claude Bernard, as well as the well-known work of Borgström et al. (1957) had demonstrated that fat absorption normally occurs in the proximal small intestine. The experiments cited in paper VII although incomplete because of the mechanical difficulty of collecting adequate samples from the distal small intestine in man, provided evidence that bile salts were absorbed more distally than fat.

These experiments raised fundamental questions regarding the mechanism of passage of micellar fatty acid and monoglyceride into the mucosal cells. Thus, the bile salt micelle could transport its lipid to the cell (or to the membrane of the microvillus) without ever being incorporated into the membrane; or, alternatively, the entire micelle might pass through the membrane into the center of the microvillus. A more attractive hypothesis is that the bile salt micelle becomes an actual part of the membrane of the

microvillus, by a process which might be termed "liquid crystalline phase coalescence" (Lawrence, personal communication). Since the bile salt-monoglyceride-fatty acid micelle is transformed into a liquid crystalline aggregate merely by removal of the water around it, it is attractive to postulate a fusion of the micelle and the liquid crystalline phase, as for example, the hexagonal cylinder (with pores) demonstrated by Luzzatti and Husson (1962). In such a mechanism, there may be an acceptor in the core of the microvillus for the fatty acid and monoglyceride which would be responsible for their movement from this hypothetical liquid crystal phase composed of micellar components and microvillus membrane to the center of the microvillus. Bile salts might then be spontaneously ejected out into the aqueous phase. No reasonable chemical or physical form has been proposed for the passage of fatty acid and monoglyceride from the microvillus membrane to the cytoplasm of the mucosal cell, where triglyceride synthesis presumably occurs. There is little experimental evidence bearing on this entire problem, although Johnston and Borgström (1964) have shown that bile salts are absorbed to isolated microvilli in vitro.

The clinical implications deriving from the demonstration of the ileal locus of bile salt absorption have been discussed (Hofmann, 1964b). Interruption of the entero-hepatic circulation by ileectomy or the administration of bile acid binding resins will greatly reduce the amount of bile salt available during fat digestion. The recirculation experiments in paper VII confirmed the previous estimate of Borgström et al. (1957) that the total bile salt pool (4 gm in man) circulated at least twice during a meal. Daily circulation is then at least 24 gm/day and since the daily synthetic rate is estimated at not more than .5 gm/day (Lindstedt, 1962), the efficiency of bile salt absorption in the steady state approaches 98%. There is now a reasonable explanation for the steatorrhea which follows distal small intestinal resection, even though fat is normally absorbed in the proximal small intestine. If bile salt is not absorbed, the amount of bile salt available for any one meal is less than 5% of normal, even if the synthesis rate is increased fourfold. Therefore, the concentration of bile salts in the lumen would be below the critical micellar concentration.

Another question of great interest is the fate of the counter ions associated with the bile salt micelle, including both those associated with the fatty acid anions and those associated with the bile salt anions. The frequent hypocalcemia and hypomagnesemia observed after distal small intestinal resection suggests that bile salts are important in divalent cation balance (see, for example, Lengemann and Dobbins, 1958).

SUMMARY

The intraluminal phase of fat digestion and absorption in man was studied by experiments with model systems containing bile salts and various lipids and by intestinal intubation experiments on human subjects.

Methods for the purification of unconjugated bile acids, the conversion of cholic acid to chenodeoxycholic acid, and the synthesis of glycine and taurine conjugates are described. Useful solvent systems for thin layer chromatographic separation of bile acids and other lipids were developed. Hydroxy-apatite was found to be a valuable adsorbent for lipid separations; 1- and 2- monoglycerides were resolved.

The solvent properties of dilute bile salts solutions were studied for a model non-polar solute, azobenzene, and a model polar solute, glycerol 1-monooleate. The critical micellar concentration for these model solutes of each of the bile salt conjugates occurring in man was measured. The saturation ratio (moles of micellar azobenzene or glycerol 1-monooleate/mole of micellar bile salt) was calculated. Bile salts were poor solvents for non-polar solutes such as azobenzene or cholesterol, unless an appropriate polar lipid was also present as a micellar solute. However, their solutions were remarkable solvents for polar lipids such as monoglyceride and lecithin.

Bile salt solutions were compared to those of typical anionic detergents and observed to be far more resistant to precipitation by added calcium ions and to possess a Krafft point well below 0°. The low Krafft point of bile salts appeared to be related to the 5 β configuration as well as the number of hydroxy substituents. The influence of pH, calcium ion concentration, and temperature on micellar aggregation was discussed. The binding of counter ions was considered.

The behavior of physiologically important solutes in bile salt solution was examined. Triglycerides and diglycerides possessed negligible solubilities, even if a polar additive was present. At appropriate experimental temperatures, all monoglycerides possessed high micellar solubilities in bile salt solutions; their behavior and solubility was described by phase diagrams. The behavior of fatty acids in bile salt solution was studied

at acid, intermediate, and alkaline pH to clarify the influence of fatty acid ionization. At acid pH, appreciable solubility of long chain saturated fatty acid was only obtained by the addition of appropriate polar lipids. At alkaline pH, all soaps had high solubility in bile salt solution because the Krafft point of bile salts was so low. The Krafft point of bile salt-soap mixtures was measured. Titration experiments indicated that the bile salt micelle enhances the ionization of solubilized fatty acid, and thus that the fatty acid in the bile salt micelle was partially ionized in vivo. A mechanism for fatty acid solubilization was proposed which provides for high fatty acid solubility at 37° and is somewhat independent of pH.

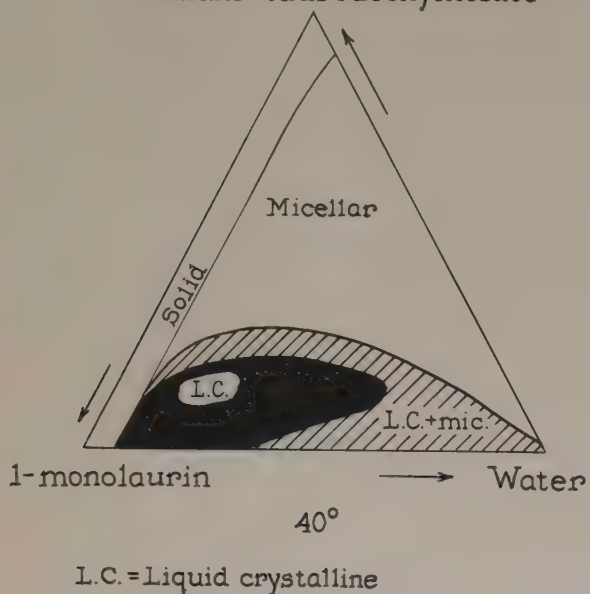
1-Monoglyceride, when present as a micellar solute, was a conveniently prepared substrate for pancreatic lipase. Experiments demonstrated directly the positional specificity of pancreatic lipase.

Subjects were fed a fat meal and intestinal content was obtained by intubation. Ultracentrifugation resolved the fluid into an upper oil phase containing higher glycerides and fatty acid and a lower, transparent micellar phase containing bile salt, fatty acid, and monoglyceride. Perfusion experiments in man demonstrated that fatty acid and monoglyceride were absorbed from a micellar solution.

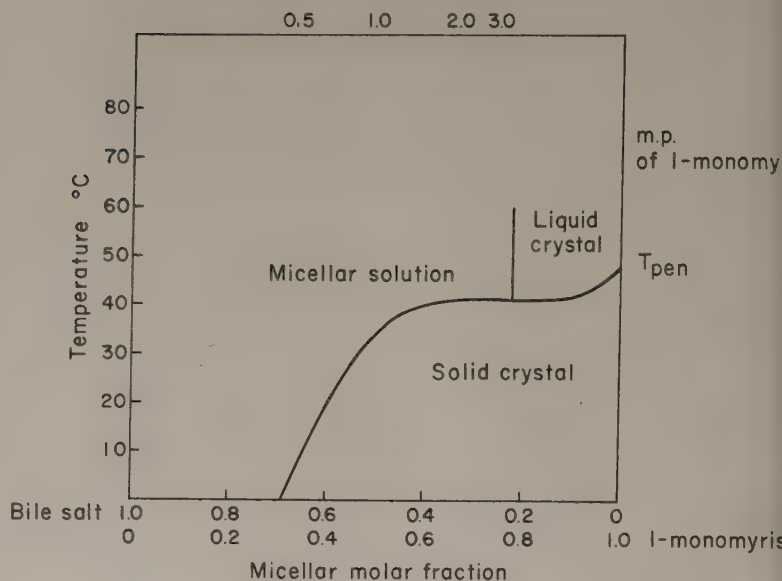
Experiments with labeled bile salts indicated that bile salts were absorbed at a more distal intestinal locus than fat.

The results indicated that a micellar phase of bile salt, fatty acid, and monoglyceride can be formed in vitro and exists in vivo. It is proposed that the bile salt micelle is the final common path of water insoluble molecules during digestion and absorption.

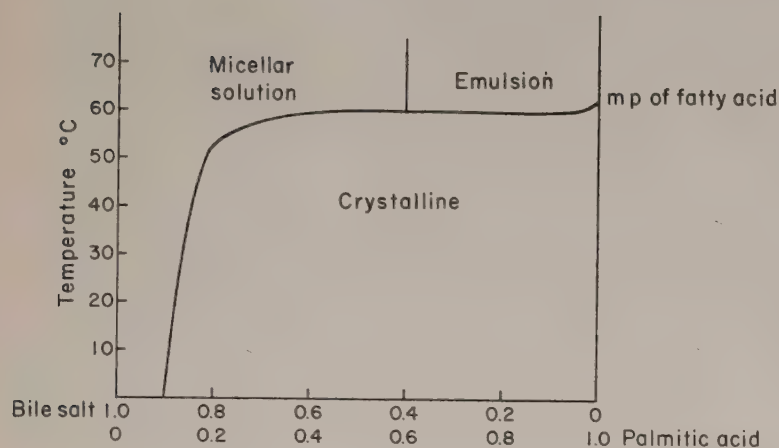
Sodium taurodeoxycholate



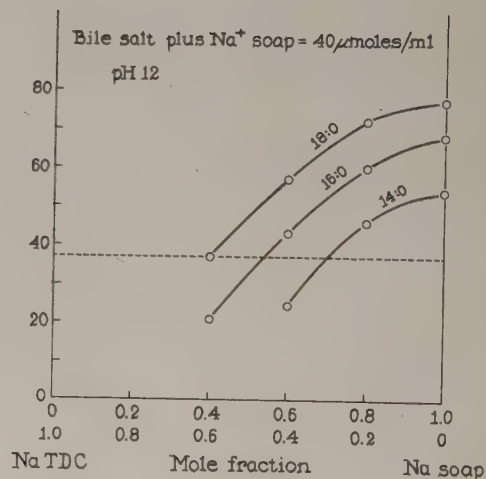
2

$$\text{Saturation ratio} = \frac{(\text{monoglyceride mic})}{(\text{bile salt mic})}$$


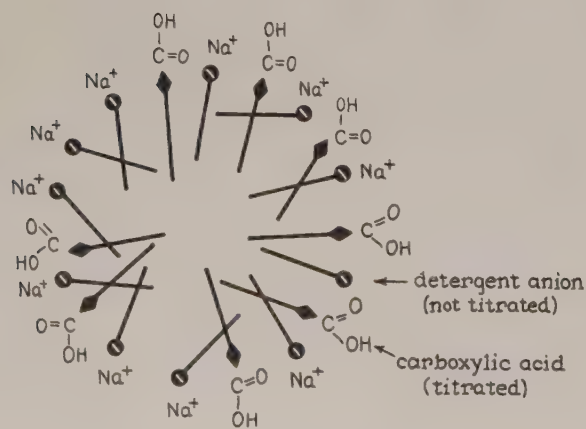
3



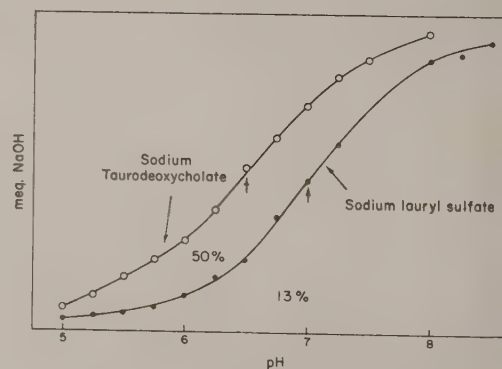
4



5



6



Solution always homogeneous, isotropic
Measurement of $pK_{a(mic)}$ = bulk pH at which fatty acid is 50% ionized

Legends for figures

Fig. 1. Composition phase diagram of sodium taurodeoxycholate-glyceryl 1-monolaurate-water (Lawrence, personal communication). The liquid crystalline phase (L.C.) is indicated in black. The diagram differs from typical soap-amphiphile-water phase diagrams by (1) the absence of a l.c. phase with soap (i.e. sodium taurodeoxycholate) and water alone, (2) the presence of a l.c. phase with amphiphile and water alone, and (3) the large micellar area. With higher monoglyceride homologues, the micellar area would be smaller and the liquid crystalline area correspondingly larger.

Fig. 2. Approximate temperature-composition phase diagram for glyceryl 1-monomyristate in sodium taurodeoxycholate solution. The sum of the concentration of sodium taurodeoxycholate and glyceryl 1-monomyristate was 40 mM. Since only six ratios of components were prepared, a eutectic point may have been missed. The micellar area (and hence the saturation ratio) increases markedly at about 41°. With higher monoglyceride homologues, the micellar area would be smaller and the temperature at which the marked increase in micellar area occurs, would be higher.

Fig. 3. Approximate temperature-composition phase diagram for palmitic acid in sodium taurodeoxycholate solution; the sum of the components is 40 mM and the pH, 4.0. A marked increase in the micellar area occurs over a narrow temperature range. This temperature corresponds to the m.p. of the fatty acid in bile salt solution or water.

Fig. 4. The Krafft points of sodium taurodeoxycholate and sodium soap mixtures. (18:0, stearate; 16:0, palmitate; and 14:0, myristate). The sum of the components was 40 mM; the pH, 12. At 37°, indicated by the dotted line, the saturation ratios of myristate, palmitate, and stearate are 2.5, 1.3, and 0.7 respectively.

Fig. 5. Schematic representation of the titration of a fatty acid present as a micellar solute in a detergent solution. The pK_a of the detergent must be sufficiently different (either higher or lower) that the detergent anions remain completely ionized during the entire titration of the solubilized carboxylic acid.

Fig. 6. Titration of oleic acid, when solubilized in micellar solution of sodium taurodeoxycholate or sodium lauryl sulfate at 37°. The detergent concentration was 16 mM; the fatty acid concentration, 5 mM; therefore the ratio of micellar oleic acid to micellar detergent was about .3. The vertical arrows indicate the bulk pH corresponding to half ionization of the fatty acid. The figures indicate the percentage of fatty acid ionized at pH 6.3, a pH corresponding to that present in intestinal content.

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